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True Hermaphroditism in a 46,XY Individual, Caused by a Postzygotic Somatic Point Mutation in the Male Gonadal Sex-determining Locus (SRY): Molecular Genetics and Histological Findings in a Sporadic Case

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Summary

Recently, the gene for the determination of maleness has been identified in the sex-determining region on the short arm of the Y chromosome (SRY) between the Y-chromosomal pseudoautosomal boundary (PABY) and the ZFY gene locus. Experiments with transgenic mice confirmed that SRY is a part of the testis-determining factor (TDF). We describe a sporadic case of a patient with intersexual genitalia and the histological finding of ovotestes in the gonad, which resembles the mixed type of gonadal tissue without primordial follicle structures. The karyotype of the patient was 46,XY. By PCR amplification, we tested for the presence of PABY, SRY, and ZFY by using DNA isolated from peripheral blood leukocytes and for the presence of SRY by using DNA obtained from histological gonadal slices. The SRY products of both DNA preparations were further analyzed by direct sequencing. All three parts of the sex-determining region of the Y chromosome could be amplified from leukocytic DNA. The patient's and the father's SRY sequences were identical with the published sequence. In the SRY PCR product of gonadal DNA, the wild-type and two point mutations were present in the patient's sequence, simulating a heterozygous state of a Y-chromosomal gene: one of the mutations was silent, while the other encoded for a nonconservative amino acid substitution from leucine to histidine. Subcloning procedures showed that the two point mutations always occurred together. The origin of the patient's intersexuality is a postzygotic mutation of the SRY occurring in part of the gonadal tissue. This event caused the loss of the testis-determining function in affected cells.

Introduction

The recent developments in molecular genetics have ascribed the testis-determining factor (TDF) responsible for the induction of testicular structures in the primordial gonad to a so-called sex-determining region of the Y chromosome (SRY) located about 8 kb proximal to the Y-chromosomal pseudoautosomal boundary (PABY; Ellis et al. 1989; Sinclair et al. 1990). The SRY has the characteristics of a transcription factor, codes for a highly conserved amino acid sequence, and is specific for the Y chromosome in all mammals tested to date (Gubbay et al. 1990; Koopman et al. 1990; McLaren 1990).

Another gene, previously thought to be the TDF, encodes a zinc-finger protein (ZFY) and is located about 160 kb from the PABY (Page et al. 1987). It seems to be an important factor in regulating sex-determining mechanisms, but deletion studies have shown that it is not the main factor regulating sex determination (Koopman et al. 1989; Palmer et al. 1989).

Most of our knowledge regarding the function of the Y chromosome in man is derived from the detailed molecular analysis of sex-reversed XX males and XY

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females, as well as of true hermaphrodites. In sex-reversed XY females gonadal dysgenesis with unambiguous external female genitalia are the prominent clinical features (Swyer 1955). The first results of molecular genetics have shown that there are SRY-negative XY females, and sequence analysis in SRY-positive XY females revealed point mutations or microdeletion in the three patients published so far (Disteche et al. 1986; Affara et al. 1987; Levilliers et al. 1989; Berta et al. 1990; Ferguson-Smith et al. 1990*b*; Jäger et al. 1990). However, point mutations causing a conservative amino acid substitution do not necessarily lead to a sex-reversed phenotype (Vilain et al. 1992).

Sex-reversed XX males can be distinguished into two groups on the basis of clinical grounds and the presence or absence of Y-chromosome material (de la Chapelle 1972, 1987; Nakagone et al. 1991). Y-positive XX males show features similar to those of Klinefelter patients, while Y-negative males resemble true hermaphrodites (Ferguson-Smith et al. 1990*a*). Furthermore, sex-reversed XX males have been identified in the same pedigree as have 46,XX true hermaphrodites (Rosenberg et al. 1963; Kasdan et al. 1973; Skordis et al. 1987).

In true hermaphroditism, ovarian and testicular structures coexist in the gonads, and internal and external genitalia are often abnormally differentiated (van Niekerk 1981). The frequency of the karyotypes of true hermaphroditism varies in different populations: 46,XX is the most frequent in Europe and Africa, while 46,XY seems to be predominant in Japan (Overzier 1961; Tazaki et al. 1964; van Niekerk and Retief 1981; Aaronson 1985; Ramsay et al. 1988). Various combinations of mosaicism are found in at least 10%–20% of patients, and the development of dual gonads seems to be easily explained in these cases.

The origin of an ovotestis in patients with an uneqivocal karyotype of 46,XX or of 46,XY is more difficult to explain, in particular if no translocation of Y material is found (Pereira et al. 1991). True hermaphroditism with a karyotype of 46,XX and sex-reversed XX males (both negative for SRY) may coexist in the same family. A common origin of these disorders of male sex differentiation can, therefore, be assumed (Abbas et al. 1990). In this case, testicular tissue develops without detectable SRY. Therefore, further studies are needed to elucidate the molecular mechanisms of sex differentiation and the role of the SRY.

True hermaphrodites with a karyotype of 46,XY are rare and are mostly observed in sporadic cases (Librik and Clayton 1972). At present, no data on molecular genetics have been reported. It is not clear whether a common defect also may lead to the development of sex-reversed XY females as well as of 46,XY true hermaphrodites, as must be assumed in the family with XX males and 46,XX true hermaphrodites.

We report here our examination of three Y chromosome–specific regions in a 46,XY true hermaphrodite: the PABY, the SRY locus, and the last exon of the ZFY gene. We are able to correlate these results with the histology of the ovotestis, and thus we are able to provide an explanation for the pathology of the gonadal development.

Patient and Methods

Description of the Patient

The patient was admitted to our hospital after birth (1978) for evaluation of ambiguous genitalia. The parents are of southern German origin, and there is no consanguinity in the family.

External genitalia showed an enlarged clitoris (~1 cm) and a urethral orifice ending at the base of the clitoris. Two gonads were palpable in the labioscrotal folds. Genitography showed a hypoplastic vaginal pouch, but no uterus was identified. The karyotype was 46,XY. The child was given a female sex assignment, and, after exclusion of enzyme defects, both gonads were extirpated at the age of 1½ years.

Histological Preparation

The specimen obtained from one labioscrotal fold was 2 cm × 1.5 cm × 0.7 cm in size and had been fixed in formalin. After macroscopical preparation, the tissue had been routinely embedded in paraffin, and 5-μm sections had been prepared. After being dewaxed, the sections had been stained with hematoxylin-eosin (HE), perjodacid-Schiff-reaction, and v. Gieson. Multiple 30-μm sections were prepared for PCR from the paraffin block, kept since 1980 in the archive. No material was left for further histological, immunohistochemical, or ultrastructural studies.

Preparation of DNA

Genomic DNA from leukocytes was prepared from 10-ml EDTA blood samples collected from the patient and the parents. After centrifugation of the whole blood sample, the plasma supernatant was discarded. The erythrocytes were lysed twice in ice-cooled isotonic ammonium buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA pH 7.4). The white blood cells were resuspended in 5 ml of 75 mM NaCl and 25

mM EDTA pH 8.0, were lysed with 250 µl of 20% SDS solution, and were incubated with 150 µg of Pronase E (Sigma, Munich) at 37°C for 14 h. The sample was vortexed with 1.5 ml of 5 M NaCl for 10 s and centrifuged for 10 min at 3,000 *g*. The supernatant DNA was precipitated with 20 ml of 100% ethanol and was washed in 10 ml of 70% ethanol. The precipitated DNA was resuspended in 0.5 ml of 10 mM Tris/HCl and 1 mM EDTA pH 8.0.

Genomic DNA from histological slides was prepared as follows: after the tissue slices (30 µm thick) were scraped from the slides, they were deparaffined twice in 1 ml of Xylol for 30 min. Rehydration was done by using descending ethanolic concentrations (96%, 90%, and 70%, each for 15 min). Thereafter, the tissue was treated with Proteinase K in a 50 mM Tris/HCl pH 7.5, 10 mM EDTA, 150 mM NaCl, and 1% SDS buffer at 55°C for at least 12 h. The DNA was treated once with phenol/chloroform/isoamylethanol (25/24/1) and twice with chloroform alone. The DNA was precipitated with 1 vol propan-2-ol and was washed with 70% ethanol. The pellet was dissolved in 20 µl of 10 mM Tris/HCl and 1 mM EDTA pH 8.0.

PCR

For PCR, the following specific oligonucleotides were used: SX1 (5′-GCTCTCCGGAGAAGCTCTTC-3′), SX2 (5′-GATAGAGTGAAGCGACCCATG-3′), SX3 (5′-CATCTTCGCCTTCCGACGAGG-3′), and SX8 (5′-CTGTAGCGGTCCCGTTGC-3′) for the SRY-conserved motif (SX1, SX2, and SX3 were designed from the SRY sequence published by Sinclair et al. [1990]; and SX8 was deduced from the SRY cDNA sequence [A. Braun, unpublished data]); ZFY1 (5′-AAAAATTCATGAGGAGAC-3′) and ZFY2 (5′-AAGCTTGTAGACACAC-3′) for the last coding exon of the ZFY gene were designed from sequence data published by Schneider-Gädicke et al. (1989); and BXY1 (5′-GCTTTGAAGGAACTCTGC-3′) and BXY2 (5′-AGAGCTTATATACCTTC-3′) for the pseudoautosomal boundary of the Y and X chromosomes were designed from the sequence published by Ellis et al. (1989). The control PCR with DNA from tissue slices was performed with primers—A17 (5′-CCTGTGAAAGTAATTCTCC-3′) and A18 (5′-TTCCTTTGGATCTTTCCTG-3′)—for exon 4 of the vitamin D–binding protein (chromosome 4). The *Taq* DNA polymerase purchased from Promega used leukocytic DNA as template, and that purchased from Amersham used DNA from gonadal tissue as template. The total reaction volume of 40 µl included about 1 µg of leukocytic DNA or 5 µl of gonadal DNA, 50 ng of each primer, 1.25 U of *Taq* DNA polymerase, 200 µmol of each dNTP, and 1.5 mM magnesium chloride. Each sample of genomic DNA was subjected to 30 amplification cycles, and DNA of histological slices was subjected to 40 amplification cycles of 1 min at 94°C for denaturation, 1 min at either 57°C (SRY), 50°C (ZFY), 45°C (PABY and PABX), or 53°C (exon 4 of DBP) for annealing, and either 1 min (ZFY) at 72°C or 30 s (for all others) at 72°C for extension. Initial denaturation was done at 94°C for 7 min. Booster reactions (30 cycles) were performed with PCR products of gonadal-slice DNA preparations by using 1 µl of the first PCR.

Agarose Electrophoresis

Either 10 µl (SRY), 15 µl (ZFY), or 20 µl (PABY and PABX) of the PCR products obtained from genomic DNA and 20 µl of the PCR products obtained from DNA of the histological slices were separated in 1% agarose gels. The electrophoresis buffer was 85 mM Tris, 90 mM boric acid, and 2 mM EDTA, with ethidium bromide (0.35 µg/ml). One hundred volts were employed for 1.5 h. The separation was directly visualized by UV fluorescence at 302 nm and was immediately photographed on Polaroid film. For all agarose gels the 1-kb ladder supplied from BRL was used as size standard.

DNA Hybridization

Agarose gels were blotted overnight on Hybond N^+ (Amersham) in the presence of 0.4 N NaOH. Specific probes, which were gel-purified leukocytic PCR products, were radiolabeled by PCR. The reaction buffer described above was used, with the exception that the final concentration of each nucleotide including [α-^{32}P]dATP (Amersham) was 10 µmol. Hybridization conditions were done according to a method described elsewhere (Braun et al. 1992).

Subcloning of SRY

SRY-specific products from the first PCR reaction of the DNA from gonadal slices were subcloned in pUC 19. The subcloning procedure, screening, and preparation of plasmid DNA for sequencing were done according to methods described elsewhere (Braun et al. 1992).

DNA Sequencing

Sequencing of plasmids was performed by the dideoxy chain-termination method using the T7 polymerase sequencing kit, supplied by Pharmacia, according to

the manufacturer's instructions. For direct sequencing we used the method described by Casanova et al. (1990). PCR products were purified with the help of Quiagen's PCR purification kit, and about 300 ng of DNA was denatured at 95°C for 5 min. Annealing was done in the presence of 2.5 ng of SRY-specific primers at −70°C for 30 s. Sequencing was performed with the above-mentioned kit: labeling time was 3 min, and termination time was 4 min. Sequencing products were labeled by using [α-^{32}P]dATP. The sequencing electrophoresis was done with a 6% acrylamide 6 M urea gel in a DNA sequencing electrophoresis unit (21 cm × 50 cm). The running conditions were constant 2,000 V at 50°C for 2 h.

Results

Histology of the Gonad

Some ductuli efferentes of the caput epididymidis and partial ductus deferens were found. The testicle measuring 0.7 cm in maximal diameter was composed of rete testis and immature seminiferous tubules surrounded by slightly fibrous, thickened tunica albuginea. In the interstitium, no Leydig cells could be identified. The tubules were surrounded by an intact basement membrane. Large cells with a diameter of about 25 μm, showing pale cytoplasma and delicate chromatin of the round nuclei with small nucleoli, were disseminated beside Sertoli cells and spermatogonia and were found predominantly in the peripheral region; these cells resemble oocytes (fig. 1). Complete primordial follicles with follicle epithelium were not identified. The histological pattern seems similar to the mixed type of gonadal tissue described by McKelvie et al. (1987) as a rare form of ovotestis in true hermaphroditism.

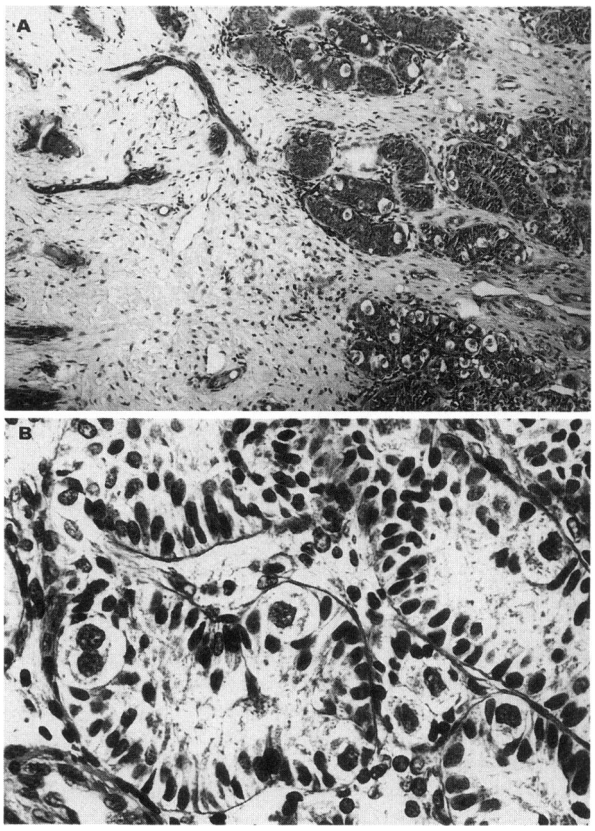

Figure 1 Histology of the ovotestes. *A*, Gonadal tissue with rete testis (left side) and immature seminiferous tubules with disseminated cells resembling oocytes (large white cells). Paraffin section is from 1980 and is stained with HE (magnification ~120×). *B*, Immature seminiferous tubules with disseminated large, pale cells. These oocytes resembling cells have distinct cell membranes but no follicle epithelium. Paraffin section is from 1980 and is stained with HE (magnification ~500×).

PCR with DNA from Peripheral Blood Leukocytes

Our examination focused on three sites on the short arm of the Y chromosome: the PABY, the SRY locus, and the ZFY locus. We could amplify all three loci from the patient's and the father's DNA (fig. 2). The amplification product of the psuedoautosomal boundary consists of two specific bands, with a size of 221 bp for the X-specific boundary and a size of 531 bp for the Y-specific boundary. The SRY-specific product was 517 bp, and the last exon of the ZFY was 1,275 bp in length. The mother's DNA preparation was positive for the X-chromosomal boundary only; all other amplification reactions yielded no specific products. Sequences of the SRY-specific product of the patient and the father were identical to the sequence published in the literature (Sinclair et al. 1990).

PCR with DNA from Histological Slices of the Ovotestis

We established the PCR with DNA from histological slices, by amplification of 179 bp of the exon 4 from the vitamin D–binding protein gene, which is localized on chromosome 4 in humans (Cooke et al. 1986). The PCR product can be barely recognized in the agarose gel (fig. 3C, A), but, in the following Southern blot, a clear signal was seen on the autoradiography when the PCR product hybridized with a specific probe (fig. 3C, B). By using PCR primers for the DNA sequence of the conserved 80-amino-acid motif of the SRY locus, the 240-bp product was amplified and seen in the agarose gel

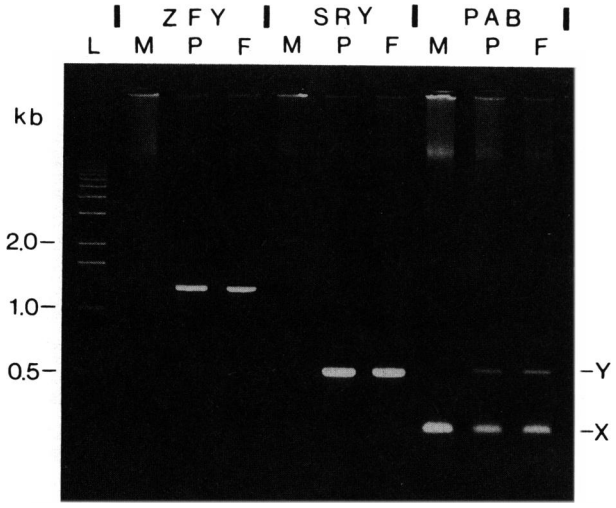

Figure 2 Ethidium bromide–stained agarose gel (1%) with the PCR products from either ZFY, SRY, or PABX/PABY. Genomic DNAs from peripheral blood leukocytes were used for PCR with specific primers ZFY1/ZFY2 for ZFY, SX1/SX8 for SRY, and BXY1/BXY2 for PABX or PABY, respectively. The patient (lanes P) shows a male-specific pattern for all three loci, which resembles that of the father (lanes F). The PCR with DNA from the mother (lanes M) results only in an X-chromosomal specific band by using primers for the pseudoautosomal region. The numbers at the left indicate the size, in kilobases (kb), of bands of the BRL 1-kb ladder (lane L).

(fig. 3*SRY*, A) and was clearly demonstrated with a specific probe on the Southern blot (fig. 3*SRY*, B).

Sequencing of the Gonadal SRY-specific PCR Product

Direct sequencing of the gonadal SRY-specific PCR product of the patient revealed two mutations. One mutation was in the codon for amino acid 22 of the conserved motif. The last nucleotide of the codon is mutated by a transition from thymidine to cytosine (fig. 4*A*). This mutation is silent; the amino acid coded remained alanine. The second mutation was in the codon for amino acid position 44; here, the second nucleotide of the codon is transversed from thymidine to adenosine (fig. 4*B*). This mutation resulted in a nonconservative amino acid substitution from leucine to histidine. The DNA preparation starting from gonadal tissue, PCR, booster PCR, and sequencing was reproduced three times, with uniformly identical results. Thus failures in DNA preparation or *Taq* polymerase amplification were excluded. Figure 5 summarizes the results of both mutations, according to the published sequence of the SRY motif (Sinclair et al. 1990).

In order to examine whether the two mutations occurred either together in the same DNA molecule, i.e., in the same cell, or independently from one another, subcloning procedures were performed. Figure 6 shows two of five of the subclones tested, demonstrating that the two mutations are either together in the sequence or absent; that is, the wild type is present (*A* = mutant, and *B* = wild type).

Discussion

The patient described in this report is the first true hermaphrodite with a karyotype of 46,XY in whom the pathogenesis of dual gonadal development could be ascribed to a mutation in the SRY. In the gonadal tissue —besides the wild type—two point mutations could be identified, which can be seen as a double band at the same position in the sequencing gel. While one mutation was silent, the second exchange of base pairs led to

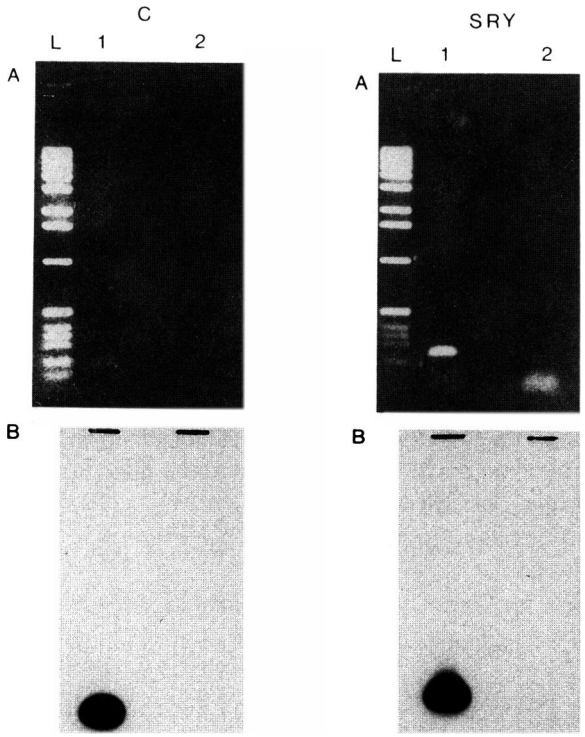

Figure 3 PCR products obtained by using genomic DNA prepared from the histological slices of the patient's ovotestes. Shown are (*A*) ethidium bromide–stained agarose gels (1%) and (*B*) Southern blots of the agarose gels, hybridized with specific probes for exon 4 of the vitamin D–binding protein gene as control (C) or *SRY*, respectively. Lanes 1, Specific PCR, including all components. Lanes 2, Contamination control of the substances used in the PCR excluding DNA from the tissue. The size of the control product is 179 bp. The size of the SRY product is 240 bp. Lanes L, 1-kb-ladder size standard (BRL).

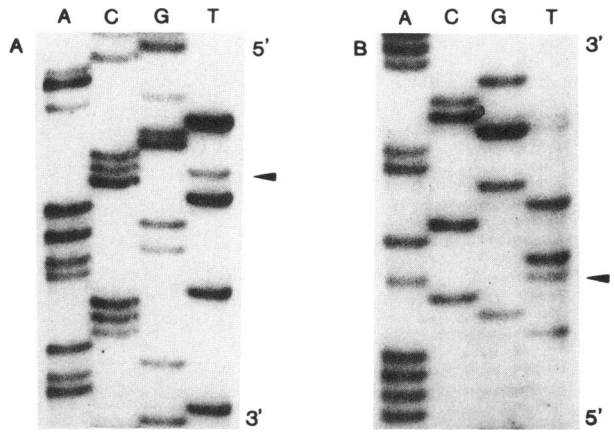

Figure 4 Part of the sequence from the SRY in the ovotestes. *A*, DNA sequence around the triplet coding for amino acid 22 of the 80-amino-acid conserved motif of the SRY. Thymidine is the nucleotide in the wild type, and cytosine is the nucleotide in the mutant SRY. It is a silent mutation because the triplets GCT and GCC both code for alanine. *B*, DNA sequence around the triplet coding for amino acid 44 of the conserved motif. Thymidine is the nucleotide in the wild type, and adenine is the nucleotide in the mutant SRY. This mutation caused a nonconserved amino acid substitution from leucine to histidine in the gene product.

an altered translation product with the loss of information, i.e., the loss of biological function of the TDF. The final result is the incomplete induction of testicular tissue in part of the gonadal tissue. The SRY sequence identified in the father and in the leukocytic DNA of the patient was identical to the sequence published in the literature (Sinclair et al. 1990). Neither in the father's nor in the patient's leukocytic DNA could mutant SRY be identified; furthermore, the mutant SRY was present in only part of the gonadal tissue. This indicates that the mutation occurred during the early embryonic development.

To exclude the possibility that the two point mutations are due to a *Taq* polymerase infidelity, we repeated the procedure starting from DNA preparation from gonadal tissue (each preparation comprised eight slices), followed by PCR, booster PCR (together 70 cycles), and sequencing three times; the results were identical. It can be excluded that the *Taq* polymerase produced failures at identical sites of the DNA sequence in three separate preparations.

The presence of mutant and wild-type SRY might explain the development of an ovotestis in this patient. The most commonly reported histology of the ovotestis of true hermaphrodites shows a clear demarcation line between ovarian and testicular tissue, and usually an end-to-end fusion of ovary and testis is described (van Niekerk 1981; van Niekerk and Retief 1981). In contrast, the histological picture in our patient showed a diffusely distributed mixture of presumably ovarian cells within testicular tubules, a finding rarely reported in true hermaphroditism (McKelvie et al. 1987). It is intriguing to speculate that this mixture of ovarian and testicular cells provides evidence that an intact SRY is needed in each single cell of the gonad in order to initiate normal testicular structures in the primordial gonad. Further studies correlating histology and molecular findings are needed to explain these results.

The two point mutations are always found together in the same subclones, i.e., in the same cells. This fact indicates that they have originated together and that they were caused by the same mutagenic agent. There was no history of medical X-ray exposure of the mother during the pregnancy with this child. However, during early pregnancy the mother worked in a chemical factory as a technician experimenting with various chemicals, such as organic solvents, for the impregnation of leather and fabrics. The cause of the two point mutations in this case is unclear.

Mutations in the SRY have been published so far for three XY females; two point mutations and a deletion in the SRY of the leukocytic DNA were described

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                SX 2 >
GTC CAG GAT AGA GTG AAG CGA CCC ATG AAC GCA TTC ATC GTG TGG TCT
Val Gln Asp Arg Val Lys Arg Pro Met Asn Ala Phe Ile Val Trp Ser

                          Ala
                           C
CGC GAT CAG AGG CGC AAG ATG GCT CTA GAG AAT CCC AGA ATG CGA AAC
Arg Asp Gln Arg Arg Lys Met Ala Leu Glu Asn Pro Arg Met Arg Asn

                                                   His
                                                    A
TCA GAG ATC AGC AAG CAG CTG GGA TAC CAG TGG AAA ATG CTT ACT GAA
Ser Glu Ile Ser Lys Gln Leu Gly Tyr Gln Trp Lys Met Leu Thr Glu

GCC GAA AAA TGG CCA TTC TTC CAG GAG GCA CAG AAA TTA CAG GCC ATG
Ala Glu Lys Trp Pro Phe Phe Gln Glu Ala Gln Lys Leu Gln Ala Met

CAC AGA GAG AAA TAC CCG AAT TAT AAG TAT CGA CCT CGT CGG AAG GCG
His Arg Glu Lys Tyr Pro Asn Tyr Lys Tyr Arg Pro Arg Arg Lys Ala

 < SX 3
AAG ATG CTG CCG AAG AAT TGC AGT TTG CTT CCC GCA GAT CCC GCT TCG
Lys Met Leu Pro Lys Asn Cys Ser Leu Leu Pro Ala Asp Pro Ala Ser
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Figure 5 Presentation of the SRY's conserved motif of 80 amino acids, including the mutations in the patient. One of the two mutations concerns amino acid 22 of the conserved motif. However, it does not result in an amino acid substitution. The triplet GCT, as well as the triplet GCC, codes for the amino acid alanine. The second mutation, at position 44 of the SRY's motif, produces a nonconserved amino acid substitution from leucine (CTT) to histidine (CAT). The distance from mutation 1 to mutation 2 is 66 bp, which constitutes 6½ rounds of the double-helical DNA molecule. The primers for PCR amplification starting with DNA from histological slices are underlined.

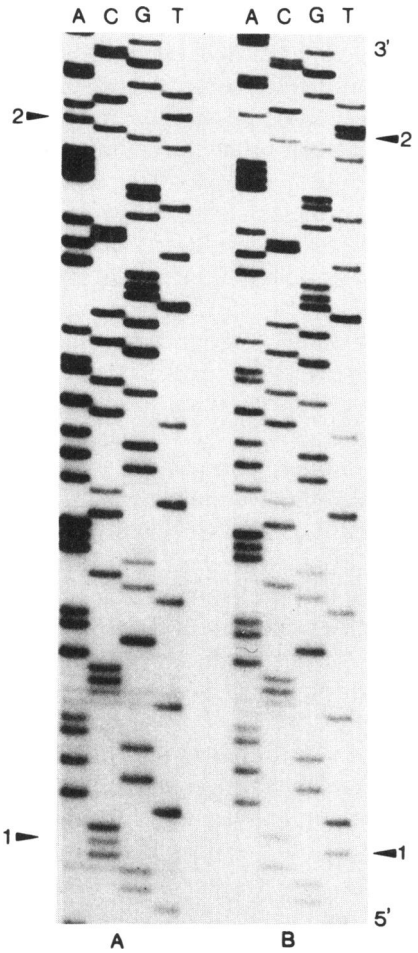

Figure 6 DNA sequences of two subcloned SRY PCR molecules. *A*, DNA sequence of one subclone which bears both mutations, indicated at the left by the numerals "1" and "2." *B*, DNA sequence of a subclone with the SRY sequence of the wild type. Regions of interest are marked by the numerals "1" and "2" at the right side. Since the two mutations always occur together, it has to be assumed that they originate from the same DNA molecule.

(Berta et al. 1990; Jäger et al. 1990). In a further three-generation family a point mutation was found in the SRY open reading frame which did not show a complete penetrance; only three of five 46,XY individuals were sex-reversed (Vilain et al. 1992). In these earlier reports, the mutations must have originated in the patients before the first zygotic division, most likely in the father's germ cells. In contrast to our patient, all patients described elsewhere also developed gonadal dysgenesis with the complete loss of gonadal function and an unambiguous female phenotype. The wild-type SRY in the gonad of our patient might have prevented the development of complete gonadal dysgenesis.

Whether this mechanism, a postzygotic mutation of the SRY which is present only in part of the germ cells, is responsible for the development of a 46,XY true hermaphrodite, in contrast to a 46,XY female with complete gonadal dysgenesis, remains to be proved. However, our data provide further evidence that the SRY is at least an essential part of the TDF. The questions of why and how ovarian structures develop in the presence of a defective SRY remain unanswered.

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